A Novel Assay for Neutrophil Extracellular Traps (NETs) Formation Independently Predicts Disseminated Intravascular Coagulation and Mortality in Critically Ill Patients

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At a Glance Commentary

Scientific Knowledge on the Subject

Neutrophils are the first line of defence against bacterial infection and formation of neutrophil extracellular traps (NETs) is an important protective mechanism. However, NETs can also cause harm by exposing cytotoxic histones and promoting intravascular coagulation. Although increasingly considered as important therapeutic targets, there is currently no robust measure of NETs formation to inform clinical care and enable precision medicine in patients on the intensive care unit (ICU).

What This Study Adds to the Field

We have established a novel assay by incubating patient plasma with neutrophils to directly induce and measure NETs-formation. This is different from currently available assays, which primarily detect NETs-breakdown products. Using this assay in a prospective cohort of 341 ICU patients, we found that the degree of NETs formation is significantly associated with disease severity and independently predicted development of disseminated intravascular coagulation (DIC) and mortality. This assay also enabled identification of interleukin-8 (IL-8) as a major factor that drives NETosis through mitogen-activated protein kinase (MAPK) pathway activation. Inhibiting IL-8 or MAPK significantly reduced NETs formation. Therefore, this assay can inform on the *in vivo* capacity for NETs formation and its inducing factors to enable improved therapeutic targeting strategies for ICU patients.

Supplemental data:

This article has an online data supplement, which is accessible from this issue's table of content online at **www.atsjournals.org**

Abstract

Rational: Neutrophil extracellular traps (NETs) are important in the host defence against infection, but also promote intravascular coagulation and multi-organ failure (MOF) in animal models. Its clinical significance remains unclear and available assays for patient care lack specificity and reliability.

Objectives: To establish a novel assay and test its clinical significance

Methods: A prospective cohort of 341 consecutive adult ICU patients was recruited. The NETs-forming capacity of ICU admission blood samples was semi-quantified by directly incubating patient plasma with isolated neutrophils ex vivo. The association of NETs–forming capacity with sequential organ failure assessment (SOFA) scores, disseminated intravascular coagulation (DIC) and 28-day mortality were analysed and compared with available NETs assays.

Measurements and Main Results: Using the novel assay, we could stratify ICU patients into 4 groups with absent (22.0%), mild (49.9%), moderate (14.4%) and strong (13.8%) NETs formation, respectively. Strong NETs formation was predominantly found in sepsis ($P < 0.0001$). Adjusted by APACHE II, multivariate regression showed that the degree of NETs formation could independently predict DIC and mortality whereas other NETs assays, e.g. cell-free DNA, myeloperoxidase and myeloperoxidase-DNA complexes, could not. IL-8 levels were found to be strongly associated with NETs formation and inhibiting IL-8 significantly attenuated NETosis. MAPK activation by IL-8 has been identified as a major pathway of NETs formation in patients.

Conclusions: This assay directly measures the NETs-forming capacity in patient plasma. This could guide clinical management and enable identification of NETs-inducing factors in individual patients for targeted treatment and personalised ICU medicine.

Introduction

Morbidity and mortality rates in critically ill patients remain high despite significant advances in intensive care unit (ICU) management. Sepsis is a major driver of poor outcome and as sepsis definitions have shifted towards infection-triggered organ dysfunction (1), the pathophysiology that underlies progressive organ failure requires further elucidation (2). The microcirculation plays a key role in the development of organ dysfunction and is particularly vulnerable to the interactions between inflammation, coagulation and innate immune activation (3). Aberration of this process can cause 'immunothrombosis' (4) and promote development of disseminated intravascular coagulation (DIC) to impair microcirculation.

The role of neutrophils in immunothrombosis is increasingly recognised (4). Activated neutrophils can expel nuclear chromatin to form NETs (5, 6) in response to different pathogens (7-12), bacterial toxins (12, 13), cytokines (12, 14-17), histones (18) and activated platelets (19, 20). Mechanistically, NETs are formed through reactive oxygen species generation via the mitogen-activated protein kinase (MAPK) pathway that specifically includes mitogen-activated protein kinase kinase (MEK–extracellular-signal-regulated kinase (ERK)) signalling (21), to trigger myeloperoxidase (MPO)-mediated activation of neutrophil elastase (NE) and protein-arginine deiminase type 4 (PAD4) activation. The resultant histone citrullination leads to chromatin decondensation and the expulsion of extracellular DNA decorated with antimicrobial enzymes (NE and MPO) and histones (22). NETs can trap and kill bacteria to form a first line defence against infection. However, excessive NETs formation facilitates immunothrombosis and even DIC (23-28), to damage microcirculation and contribute to organ failure (19, 29, 30). NETs have been recognised as therapeutic targets, particularly in critical illnesses (19, 31, 32) and monitoring the levels of NETs formation in real time may benefit these patients in clinical practice.

Although NETs can be induced and monitored *in vitro* and in animal models (12, 33), this has been difficult to quantify in clinical settings. Currently, assays to monitor NETs formation are limited to invasive organ biopsy observations or through indirect measures, such as circulating cell-free DNA (cfDNA), nucleosomes, citrullinated-histone (Cit-H3), MPO, Cit-H3-DNA or MPO-DNA complexes (34-36). The clinical potential of these surrogate markers of NETs formation have been highlighted for critical illness (26, 37, 38) but do not correlate with disease severity (34-36). Furthermore, their circulating levels are unstable and subject to enzymatic degradation (18, 39-41). Therefore, a more reliable assay is urgently required. In this study, we have developed an assay to directly determine the NETs-forming capacity of patient plasma and its clinical usage has also been evaluated in a prospective cohort of ICU patients.

Methods (413)

Study Design and participants

A prospective cohort of adult patients admitted to a general adult ICU at the Royal Liverpool University Hospital (RLUH), United Kingdom, between June 2009 and June 2013 was assessed. Patients were enrolled in accordance with the protocol approved by the NRES Committee North West - Greater Manchester West and Liverpool Central (Ref: 07/H1009/64 and 13/NW/0089). Written informed consent was obtained for all participants, and daily serial blood samples were collected over the first 96 hours of ICU admission (study duration). Exclusion criteria were: transfers from other ICUs, ICU re-admissions within 30 days, pre-existing causes of neutropenia (including haematological malignancy), intravenous heparin treatment (42) or insufficient plasma preserved to effectively perform functional analysis (Figure E1). ICU admission diagnoses were verified by two independent experienced clinicians. Admission APACHE II, daily Sequential Organ Failure Assessment (SOFA) and modified SOFA scores (platelet component removed to avoid bias from thrombocytopenia) were recorded along with outcome measures including respiratory/cardiovascular support days, length of ICU stay (LOS) and 28-day mortality (from ICU admission). Sepsis was defined using the ACCP/SCCM 2001 International Sepsis Definition (1).

DIC scoring was performed daily for the first 96 hours of ICU stay, using criteria defined by the International Society for Thrombosis and Haemostasis (ISTH).(43) DIC was diagnosed when a cumulative score of ≥5 was reached from: platelet (≥100x10^3/μl=0; <100 x10^3/μl=1; <50 x10^3/μl=2), fibrinogen (≥1.0 g/L= 0; <1 g/L=1), D-dimers (no increase=0; moderate increase=2; strong increase=3) and prolongation of prothrombin time (PT) (3 seconds=0; >3 but < 6 seconds=1; >6 seconds=2) (43).

Ex vivo assay of NETs-forming capacity

Assay development was performed using a cohort of 54 sepsis patients (NHS REC ethical approval 13/WA/0353) admitted to the ICU at Aintree University Hospital and the RLUH. The capacity of patient platelet-poor-plasma to form NETs was tested by incubating patient or healthy control plasma (or serum, where indicated) (100 µL) with heterologous neutrophils ($2x10^5$) from healthy volunteers (eMethods in Supplement) or patient-specific neutrophils, where indicated for 4 hours in glass chamber slides (BD Biosciences) at 37°C in 5% CO_2. After fixation (2% paraformaldehyde, Sigma-Aldrich), extracellular DNA was stained with 10 µg/mL Propidium Iodide (PI, Sigma-Aldrich) and visualized by immunoflourescent microscopy (20x magnification unless specified). Quantification was performed by double-blinded assessment of extracellular DNA release by three experienced clinical scientists and the average percentage was used for analysis. Degree of NETs formed was categorised into 4 groups: absence of NETs = 0% neutrophils forming NETs per microscopic field, mild NETs = 1% to 25%, moderate NETs = 26% to 50%, strong NETs ≥50%, including amalgam of webs. For validation, plasma-induced NETs were stained with anti-human neutrophil elastase (NE) (SantaCruz) and anti-human MPO (Abcam) along with FITC and AF700 secondary antibodies. Mechanistic studies were performed using specific inhibitors of either PAD4 (Cl-amidine (Cambridge biolabs)), IL-8 (IL-8 mAb (R&D Systems), CXCR1/2 (Reparixin (Dempé) or AZD5069 (AstraZeneca)) or MAPK signalling (U0126 (Sigma)).

Clinical samples

Following ICU admission, surplus blood samples were collected daily from all patients for the first 96 hours, in accordance with ethically approved protocols. Measurements included whole blood cell counts, coagulation parameters, NETs-related markers and cytokines (eMethods in Supplement).

Statistical Analysis

Distributions of continuous variables were assessed by Q/Q plots, histograms and Shapiro-Wilk tests. Clinical parameters were non-parametric in nature and are presented as median and interquartile ranges [1st, 3rd quartiles]. NETs-forming capacity was analysed in two ways: (a) as continuous variables (percentage NETs per microscopic field), and (b) as categorical groups, based on degree of NETs. Differences in medians between two (Mann Whitney U test) or more groups (Kruskall-Wallis test) were assessed. For cytokine analysis and comparator NETs assays, the NETs categories were also compared to healthy controls. Chi-squared test was used for categorical variables (gender, ethnicity, presence/absence of ICU admission diagnosis, DIC and 28-day mortality) between either two or more groups. Correlation analysis utilised Spearman rank's correlation test. To test whether our NETs assay and other NETs-related markers were independent predictors of DIC and mortality, multivariable analysis of crude and adjusted odds ratios were performed (with patients adjusted for APACHE II scores). Multivariate model construction is detailed in eMethods in supplement (Table E1). Receiver Operating Characteristic (ROC) curves assessed the performance of the different parameters (using continuous variables on ICU admission) for predicting DIC and mortality. Comparison of ROC curves were performed using Delong's test using MedCalc software. All other analyses were performed using SPSS (version 22) statistical software. P value (two-tailed) <0.05 was considered statistically significant.

Results

NETs can be directly induced by incubating neutrophils with plasma or sera from patients with sepsis

We observed that NETs were directly induced by heterologous healthy neutrophils incubated with platelet-poor plasma taken from a cohort of 54 sepsis patients from 2 ICUs (Figure 1A). Typical NETs structures were observed in the wells containing certain septic plasma or serum. By contrast, NETs did not form in the wells containing plasma or serum from healthy donors (n=20) (Figure 1A), unless co-incubated with 100 nM phorbol myristate acetate (PMA), a known positive control for NETs formation. For further validation of patient plasma-induced NETs, anti-human neutrophil elastase (NE) and anti-human MPO and corresponding FITC and AF700-conjugated secondary antibodies were used and we confirmed that the typical features of NETs existed (Figure 1B). Cl-amidine, an inhibitor of peptidylarginine deiminase 4 (PAD4) and NETosis, was able to block the plasma or serum-induced NETs (Figure 1C). We compared the plasma and serum isolated from blood samples taken from the same patients at the same time and found that either plasma or serum could induce similar amount of NETs (Figure 1D). Moreover, experiments were also performed using patient-specific (n=10) neutrophils incubated with their own plasma to compare with the degree of NETs generated by normal donor neutrophils (n=10) and we found no obvious difference in NETs formation (Figure 1E). The degree of NETs formation from patient plasma (n=10) was repeatable with neutrophils isolated from different healthy donors (n=10) (data not shown). Similarly, there was no exception amongst plasma from different healthy volunteers (n=20) with none of them inducing NETs (data not shown). Differential degrees of NETs formation between septic patients (n=54) (Figure 2A) were quantified as a percentage (NETs per microscopic field, see Methods) and categorised into 4 groups: Absent

(No neutrophils forming NETs per microscopic field) (n=21), Mild (1% to 25%) (n=15); Moderate (26% to 50%) (n=10) and Strong (≥50%) (n=8) (Figure 2A). The strong NETs induced by patient plasma were equivalent to PMA-induced NETs in healthy samples, whereas patient samples with absent NETs were indistinguishable from healthy controls.

These data indicate that the assay is robust and reliable for quantification. Based on this extensive assay validation work we progressed to examine plasma taken from a large cohort of ICU patients (n=341) to examine the clinical relevance of NETosis.

Sepsis is the predominant ICU condition associated with NETs formation

In total, 341 patients meeting the inclusion criteria were recruited (Figure E1). The clinical characteristics of patients are described in Table 1. We found that in 266/341 (78%) patients, their plasma could induce NETs formation. In the remaining 75/341 (22%) patients, no NETs formation was observed (Table 1). The degree of NETs formation differed between patients: 170/341 (49.9%) mild, 49/341 (14.4%) moderate and 47/341 (13.8%) strong. The degrees of NETs formation were not associated with age, gender or ethnicity (Table 1, $P>0.05$), but strongly associated with primary diseases, in particular a diagnosis of sepsis (Table 1, Figure 2B). Two-third of moderate and strong NETs formation was induced by plasma from patients with sepsis, whilst more than 70% of mild NETs formation was induced by plasma from non-septic patients. There was no significant correlation between NETs formation and white blood cell ($R=-0.336$, $P=0.062$) and neutrophil counts ($R=-0.309$, $P=0.114$) (Table 2), or with other NETs-related markers such as cfDNA ($R=-0.134$, $P=0.864$), MPO ($R=0.327$, $P=0.204$) and DNA-MPO ($R=0.158$, $P=0.982$) (Table E2).

Levels of NETs formation in patient plasma strongly predict DIC development

High levels of NET formation were strongly associated with thrombocytopenia (platelets <150 x10^9/L) (P<0.0001). Over 60% of patients whose plasma induced moderate and strong NETs-formation had thrombocytopenia compared to 15.9% in the absent and mild groups (χ^2 test, P<0.001). Abnormality in PT, activated partial thromboplastin time (aPTT) and fibrinogen, as well as D-dimer were also significantly associated with moderate or strong NETs formation (P<0.05, compared to mild or absent groups) (Table 2). These parameters are collectively indicative of DIC (43) and indeed, DIC development was significantly higher in patients with strong (39.4%) and moderate (26.6%) NETs formation compared to mild (1.2%) and absent (4.2%) groups (χ^2 test, P<0.0001). NETs formation was significantly higher in patients with DIC (median: 50.0% [IQR: 25.0%, 88.0%]) compared to those without (5.0% [0.0%, 20.0%]) (P<0.0001). To address whether NETs formation on ICU admission could predict DIC development post admission, we excluded patients with existing DIC on ICU admission (n=28, Table 2). Univariate analysis using the continuous percentages of NETs formation demonstrated an odds ratio for DIC was 1.06 (95% CI 1.04-1.08) (P<0.0001). Using categorical data, similar results were obtained, odds ratio 14.52 (95% CI 3.76-56.06, P<0.0001) for strong and 8.12 (95% CI 2.14-30.77, P=.002) for moderate NETs formation groups (Table 3, Figure 2C (left panel)).

Levels of NETs formation are associated with multi-system organ failure and mortality

As DIC is associated with development of organ dysfunction and poor outcome, we examined the relationship between NETs and multi-organ failure. Assessment with both SOFA and modified SOFA (platelet count removed) scores showed that the degree of NETs formation was associated with organ injury throughout the study (Table 1). Patients in the moderate and strong NETs categories had higher admission SOFA scores (median: 7 [4, 11] and 9 [7, 12]) compared to absent and mild groups (SOFA 6 [4, 9] and 6 [3, 8]) (P<0.001).

SOFA scores in the moderate and strong NETs patients remained significantly elevated throughout study duration. Furthermore, patients in the strong NETs group had higher admission modified SOFA scores (median: 8 [5, 11]) compared to absent (6 [3, 8]), mild (6 [3, 8]) and moderate groups (6 [3, 10]) (P=0.002), which also remained significant throughout the study duration. Patients whose plasma induced strong NETs formation also required more cardiovascular support days compared to patients with no NETs (median:10.0 days [IQR:7.0, 17.0] vs 8.0 [5.0, 11.0]. The mortality rates in both moderate (30.6%) and strong (34.0%) NETs formation groups were higher than in absent (12.0%) and mild (15.9%) groups (P<0.003). Univariate analysis demonstrated odds ratio of 3.24 for mortality (95% CI 1.28-8.15) (P=0.013) in the moderate and 3.79 (95% CI 1.51-9.51) (P=0.005) in the strong NETs formation group (Table 3). Using continuous percentages of NETs formation data, the odds ratio was 1.02 (1.01, 1.03) (P<0.0001). As for other NETs-related assays, there was no significant association between cfDNA, MPO or DNA-MPO with mortality (Table 3).

APACHE II is a commonly used scoring system for severity-of-disease classification. We used Spearman's Rank correlation with continuous percentages of NETs formation data and found that NETs-forming capacity was significantly associated with APACHE II scores (r=0.442, P=0.013) (Table 1). However, subsequent multivariate analysis demonstrated NETs-forming capacity was independently associated with both DIC and mortality after adjustment for APACHE II (Table 3, Figure 2C). We found that NETs formation was a strong predictor of DIC (AUC=0.851, P<0.001) (Figure 2D) (Table 3). Whilst less strong in predicting mortality (AUC=0.656; P<0.001), NETs formation was comparable to both APACHE II (AUC=0.683; P<0.001) (Delong's test vs NETs; P=0.440) and SOFA scores (AUC=0.604; P=0.009) (Delong's test vs NETS; P=0.381) (Figure 2E) (Table 3).

Anti-IL-8 partially blocks patient plasma from inducing NETs

Multiple inducers of NETs have been reported, such as IL-1β, IL-6, IL-8, TNFα and extracellular histones. Using our novel assay for NETs formation and cytokine profile multiplexes, we found that in our cohort IL-8 was the only cytokine that was significantly positively associated with NETs formation (Table E3, Figure 3A), compared to a large number of negatively correlated cytokines, including IL-5, IL-9, IL-12, IL-13, IL-17, bFGF, GM-CSF and RANTES (Table E3). To functionally investigate if IL-8 was the cytokine responsible for NETs formation in our assay, IL-8 was added to normal plasma at relevant circulating concentration (100 pg/ml). Upon incubation with healthy neutrophils, NETs formation was induced (P=0.008) (Figure 3B). NETs-forming capacities of plasma from patients with sepsis (median: 57.5 [47.5, 78.8]) (n=10) were significantly attenuated by a functional anti-IL-8 blocking mAb (median: 19 [10.0, 22.5]) (P<0.001), and the clinically trialled IL-8 receptor antagonists, Reparixin (median: 7.0 [3.5, 41.3]) (P<0.001) and AZD5069 (median: 18.5 [10.0, 28.8]) (P<0.001) (Figure 3C). Mechanistically, IL-8 signalling is predominately through Ras/Raf/MAPK pathways (44) (Figure E2), which is essential for NETs formation (21). Specific inhibition of MAPK activation using an ERK inhibitor (U0126), significantly blocked IL-8 induced NETs formation in normal plasma (P=0.005) (Figure 3B) as well NETs-forming capacity of patient plasma (P<0.001) (Figure 3C). Moreover, ERK phosphorylation induced by patient plasma was also significantly reduced by anti-IL-8 mAb treatment (P<0.001) (Figure 3D). Collectively, this supports MAPK activation as the major pathway of IL-8-induced NETs formation in patients.

Discussion

We found that NETs formation could be directly induced by patient plasma and was associated with clinically relevant information on disease severity, complications and outcome in the ICU. The extent of NETs formation on ICU admission was significantly

associated with sepsis and independently predicted development of DIC and 28-day mortality. The elucidation of IL-8 as a major contributing factor to the NETs-forming capacity of patient plasma could bridge important clinical utility with biological plausibility on the role of NETs in critical illness.

NETs have been increasingly recognised in disease pathogenesis since Brinkman et al (12) described their ability to trap and kill bacteria in tissue samples from patients with infection. As NETosis represents an integral component of the regulated immune response in preventing translocation and dissemination of infection (45-47), our results lead us to speculate that dysregulated intravascular NETosis may promote platelet trapping and cause consumptive coagulopathy to impair end-organ perfusion and provoke MOF. In support of this theory, McDonald et al (48) showed NETs-induced intravascular coagulation caused widespread microvascular occlusion and MOF in several mice models of sepsis. They also found that histones did not promote platelet adhesion to NETs or production of NETs. This could be relevant to our findings that demonstrated lack of association between histone levels and NETs formation.

Our findings that moderate to strong NETs formation is commonly observed in septic patients with respiratory origin are of particular relevance to patients with pneumonia-induced Acute Respiratory Distress Syndrome (ARDS) (49). NETs formation in these patients is associated with localised alveolar inflammation and with high IL-8 levels within bronchoalveolar lavage (BAL) fluid (50).

We believe that a key strength of this study on NETs is the demonstration of how clinically relevant information links into mechanistic understanding and identification of therapeutic

strategies. Our findings are supported by Yang et al who showed that plasma from septic patients were more likely to induce NETs than non-septic patients (51). However, their findings were limited to 62 patients with no correlation to clinical outcomes. Compared to other NETs-related assays, we found that cfDNA, MPO and MPO-DNA complexes (36, 52-56) were all poorly associated with admission severity, clinical course and outcomes. This may be due to our assay directly measuring NETs-forming capacity and not being affected by NETs degradation rates, factors altering the stability of NETs breakdown products or contaminations from neutrophil respiratory burst or death of other types of cells (Figure E2). Therefore, our assay is clearly distinct from other NETs-related assays and more accurately reflects the degrees of NETs formation in patients.

A further strength of the assay is its ability to identify the driving factors for NETosis in individual patient plasma, including important signalling pathways. This could be used for determining potential targets or guiding clinical management as part of precision or personalised medicine. We identified IL-8 as an important factor promoting NETosis in some patients using this assay and targeting IL-8 by specific inhibitors (Reparixin, AZD5069 and anti-IL-8 mAb) could significantly inhibit NETs formation in these patients. Using the same assay, we also identified MAPK activation as a major pathway for IL-8 in driving NETosis in patients. Using multivariate analysis, IL-8 levels could not independently predict DIC and mortality, similar to other reported activating factors for NETs formation (IL-1β, IL-6 and TNFα) (Table 3). This is because IL-8 levels are not uniformly elevated in patients and there are unknown factors involved which remain to be identified in future studies.

Our assay does not necessarily require isolation of individual patient neutrophils because concordant results were obtained when patient plasma was incubated with either homologous (patient-specific) or heterologous (healthy individual) neutrophils. This allows flexibility of

use in clinical practice with the choice of using patient's or healthy donor's neutrophils. Since fresh neutrophils are available in most large hospitals with blood banks and this NETs assay can be easily categorized by clinical scientists (into Absent, Mild, Moderate or Strong groups), it has clear potential to be integrated into routine clinical laboratory practice. Our assay was developed in a cohort of septic patients and evaluated in a separate cohort of ICU patients. However, limitations of this study are that our results on clinical associations were obtained in a single ICU only, but our patient cohort has consistently been representative of UK Intensive Care National Audit and Research Centre data.

In summary, this study demonstrates how a simple, direct approach to understanding NETs-forming potential in the circulation could be applied clinically to identify patients at risk of DIC and poor outcomes in ICU. We have highlighted its potential as a stratification tool on ICU admission that could enable administration of early organ support or as a companion diagnostic for novel therapies that inhibit NETs formation. As NETs and platelets interact to promote intravascular coagulation and its dissemination, there is a highly persuasive rationale for targeting NETs in sepsis and DIC. Intravenous DNase has been reported to significantly reduce end-organ damage in sepsis models (48). Our finding that IL-8 is a major inducer of NETs in many critically ill patients presents an exciting opportunity for more precise therapeutic targeting by using our novel assay system with incorporation of IL-8 inhibitors.

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Figure legends

Figure 1. NETs can be directly induced by plasma or serum from septic patients.

A, Normal healthy human neutrophils were incubated with either normal plasma ± 100 nM PMA (n=20) or critically ill patient plasma (n=54) for 4 hours, and extracellular DNA was stained with Propidium Iodide (PI). Typical images are presented. **B,** NETs formation was induced by incubating normal healthy human neutrophils with critically ill patient plasma, and extracellular DNA was stained with PI, along with human neutrophil elastase (FITC; green) and human myeloperoxidase (AF700; blue) using specific antibodies. NETs formation (arrows) was visualized using confocal microscopy. C, Pre-incubation of normal neutrophils with Cl-amidine (PAD4 inhibitor) prior to treatment with septic patient plasma blocked NETs formation (n=10) (ANOVA; P<.05). **D,** NETs formation was comparable when induced by either plasma (P) or serum (S). Matched normal plasma (n=20) and serum (n=20) did not induce NETs formation when incubated with normal healthy neutrophils, unless incubated with 100 nM PMA (n=3). There were no significant difference between septic patient plasma (n=10) and serum (n=10) in inducing NETs formation (ANOVA test; P>.05). **E,** Incubating either normal neutrophils or septic patient neutrophils, with matched septic patient plasma induced comparable NETs (n=10) (ANOVA; P<.05).

Figure 2. The degree of NETs formation is associated with sepsis and poor clinical outcomes.

A, NETs formed was categorised into 4 groups based on the percentage of neutrophils forming NETs per microscopic field which were visualized using fluorescent microscopy and PI staining. Typical images are shown. **B,** NETs formation was associated with an admission diagnosis of sepsis. Plasma from normal healthy donors did not induce NETs formation when incubated with normal neutrophils (n=20). When patients (n=341) were stratified based on admission diagnosis into those without (n=198) and those with sepsis (n=143), NETs formation was significantly elevated in those patients with sepsis (ANOVA; P<.05). **C,** Multivariable analysis of crude and adjusted odds ratios (with patients adjusted for APACHE II scores) demonstrated that NETs were an independent predictor of DIC development (left panel) and 28-day mortality (right panel) (n=341). Receiver Operating

Characteristic (ROC) curves for measuring the NETs-forming capacity of patient plasma on ICU admission, for predicting DIC development (**D**) and mortality (**E**) (n=341).

Figure 3. IL-8 contributes to the NETs-forming capacity of critically ill patient plasma, which is partially blocked by anti-IL-8 and anti-MAPK treatment.

A, Quantification of circulating factors known to stimulate NETs formation (Histones, IL-1β, IL-6, IL-8 and TNFα) in patient plasma on ICU admission, demonstrated that IL-8 was elevated in patients that were able to induce NETs (ANOVA; P<.05) (n=341). **B,** Incubation of 100 pg/ml IL-8 in normal plasma with normal healthy neutrophils for 4 hours induced NETs formation compared to normal plasma alone (n=10), which was blocked by inhibiting MAPK activation with U0126 (ERKi) (n=3) (ANOVA; P<.05). **C,** Pre-incubation of normal neutrophils with either anti-IL-8 mAb (α-IL-8) (n=10), Reparixin (REP) (n=10), AZD5069 (n=10) or MAPK inhibitor U0126 (ERKi) (n=6) prior to treatment with septic patient plasma partially blocked NETs formation (ANOVA; P<.05). **D,** Western blot analysis of ERK activation (pERK/ERK ratio) in normal neutrophils incubated for 15 mins with septic patient plasma pre-incubated without (UT) or with anti-IL-8 mAb (α-IL-8) (n=3) (ANOVA; P<.05).

	Total patients	Correlation (*R* value)	Absent NETs	Mild NETs	Moderate NETs	Strong NETs	*P* value*
Total number (n)	341	-	75	170	49	47	
NETs percentage, Median [IQR]	8.0 [0.0-25.8]	-	0.0 [0.0-0.0]	8.0 [4.0-19.0] †	45.0 [37.3-50.0] †,‡	85.5 [67.0-95.0] †,‡,§	**<.0001**
Age (years), Median [IQR]	62.0 [48.0-72.0]	.276	60.0 [47.0-69.0]	62.0 [49.0-73.0]	62.0 [44.0-76.0]	61.0 [52.0-71.0]	.730
Male (n) [%]	167 [49.0%]	-	37 [49.3%]	85 [50.0%]	22 [44.9%]	23 [48.9%]	.940
White ethnicity (n) [%]	298 [87.4%]	-	62 [82.7%]	150 [88.2%]	45 [91.8%]	41 [87.2%]	.474
APACHE II score, Median [IQR]	19.0 [14.0-25.0]	.442	19.0 [13.0-23.0]	19.0 [14.0-24.0]	20.0 [15.0, 27.0]	23.0 [17.0-29.0] †,‡	**.013**
Admission diagnosis (n) [%]							
Sepsis	143 [41.9%]	-	31 [41.3%]	48 [28.2%] †	31 [63.3%] †,‡	33 [70.2%] †,‡	**<.0001**
Respiratory sepsis	48 [14.1%]	-	10 [13.3%]	16 [9.4%]	16 [32.7%] †,‡	6 [12.8%] §	**.0004**
Abdominal sepsis	50 [14.7%]	-	12 [16.0%]	21 [12.4%]	4 [8.2%]	13 [27.7%] ‡,§	**.032**
Urological sepsis	18 [5.3%]	-	6 [8.0%]	2 [1.2%] †	5 [10.2%] ‡	5 [10.6%] ‡	**.008**
Other septic location	25 [7.3%]	-	3 [4.0%]	9 [5.3%]	6 [12.2%]	7 [14.9%] †,‡	**.047**
Trauma	61 [17.9%]	-	13 [17.3%]	36 [21.2%]	6 [12.2%]	6 [12.8%]	.366
Cardiovascular	33 [9.7%]	-	8 [10.7%]	21 [12.4%]	1 [2.0%]	3 [6.4%]	.149
Respiratory	48 [14.1%]	-	10 [13.3%]	29 [17.1%]	8 [16.3%]	1 [2.1%] †,‡	.071
Gastro-intestinal	35 [10.3%]	-	9 [12.0%]	22 [12.9%]	2 [4.1%]	2 [4.3%]	.142
Renal	5 [14.7%]	-	1 [1.3%]	2 [1.2%]	1 [2.0%]	1 [2.1%]	
Central nervous system	16 [4.7%]	-	3 [4.0%]	12 [7.1%]	0 [0.0%]	1 [2.1%]	
SOFA score, Median [IQR]							
Admission	7.0 [4.0-9.0]	.521	6.0 [3.8-9.0]	6.0 [3.0-8.0]	7.0 [4.0-11.0] ‡	9.0 [7.0-12.0] †,‡,§	**<.0001**
24 hrs post admission	7.0 [4.0-10.0]	.567	7.0 [4.0-8.0]	6.0 [4.0-9.0]	8.0 [5.0-12.0] †,‡	10.0 [7.0-13.0] †,‡	**<.0001**
48 hrs post admission	7.0 [4.0-10.0]	.597	6.0 [4.0-8.0]	6.0 [3.0-8.0]	9.0 [4.0-11.0] †,‡	11.0 [7.0-13.0] †,‡,§	**<.0001**
72 hrs post admission	6.0 [3.0-10.0]	.605	6.0 [3.0-9.0]	5.0 [3.0-7.0]	9.0 [4.0-12.0] †,‡	10.5 [8.0-14.0] †,‡	**<.0001**
Modified SOFA score, Median [IQR]							
Admission	.6.0 [3.0-8.0]	.465	6.0 [3.0-8.0]	6.0 [3.0-8.0]	6.0 [3.0-10.0]	8.0 [5.0-11.0] †,‡	**.002**
24 hrs post admission	7.0 [4.0-9.0]	.483	7.0 [4.0-8.0]	6.0 [4.0-8.3]	7.0 [4.0-11.0]	8.0 [6.0-11.0] †,‡	**.001**
48 hrs post admission	6.0 [4.0-9.0]	.506	5.0 [4.0-8.0]	5.5 [3.0-7.3]	7.0 [3.0-10.0]	8.5 [6.0-11.0] †,‡,§	**<.0001**
72 hrs post admission	6.0 [3.0-8.0]	.522	6.0 [3.0-8.0]	5.0 [3.0-7.0]	7.0 [3.3-9.0] ‡	8.5 [6.0-11.0] †,‡	**<.0001**
Organ support (days), Median [IQR]							
Mechanical ventilation	6.0 [0.0-10.5]	.322	6.0 [0.0-10.5]	4.0 [1.0-10.0]	2.0 [0.0-16.0]	8.0 [3.0-14.0] ‡	.194
Cardiovascular support	8.0 [5.0-11.0]	.356	8.0 [5.0-11.0]	7.0 [3.0-14.0]	8.0 [4.0-19.5]	10.0 [7.0-17.0] †,‡	.050
Length stay (days), Median [IQR]	9.0 [5.0-17.0]	.321	8.0 [4.0-15.0]	9.0 [5.0-16.3]	7.0 [4.0-20.5]	11.0 [7.0-19.0] ‡	.144
Mortality (n) [%]	67 [19.6%]	-	9 [12.0%]	27 [15.9%]	15 [30.6%] †,‡	16 [34.0%] †,‡	**.003**

Table 1. Characteristics of absent, mild, moderate and strong NETs formation in ICU patients

* P value for comparisons of absent vs mild vs moderate vs severe NETs patients collectively. Performed using Kruskall-Wallis test for continuous variables and Chi-squared test for categorical variables. † Significant vs absent NETs patients. ‡ Significant vs mild NETs patients. § Significant vs moderate NETs patients.

	Total	Correlation (*R* value)	Absent NETs	Mild NETs	Moderate NETs	Strong NETs	*P* value*
Total number (n)	341		75	170	49	47	
Peripheral blood cell counts							
White blood cells (x10^9/L), Median [IQR]							
Admission	12.0 [7.8-18.0]	-.336	12.1 [6.8-17.5]	12.4 [8.5-18.1]	12.3 [7.0-18.9]	9.6 [5.0-18.4]‡	.145
24 hrs post admission	11.9 [8.0-17.4]	-.289	11.6 [7.9-17.7]	12.1 [8.7-17.2]	11.2 [7.7-17.4]	10.5 [5.0-18.0]	.835
48 hrs post admission	12.0 [8.5-16.4]	-.276	12.7 [8.8-15.0]	12.0 [9.2-16.4]	10.8 [7.9-18.3]	12.0 [6.0-17.0]	.523
72 hrs post admission	11.5 [8.0-16.0]	-.114	11.4 [8.0-15.0]	11.4 [9.0, 16.2]	10.4 [8.0-15.3]	12.0 [6.9-20.1]	.862
Neutrophils (x10^9/L), Median [IQR]							
Admission	10.1 [6.6-15.4]	-.309	9.8 [6.4-15.5]	10.4 [7.3-15.4]	10.2 [6.3-14.4]	8.3 [3.9-17.7]	.469
24 hrs post admission	9.5 [6.8-15.0]	-.311	9.5 [6.5-15.1]	10.5 [7.4-15.2]	8.5 [6.3-16.2]	8.2 [4.9-13.9]	.399
48 hrs post admission	9.6 [6.4-13.9]	-.348	10.2 [7.0-14.0]	9.7 [7.1-14.0]	9.0 [6.1-13.8]	8.3 [4.1-14.0]	.430
72 hrs post admission	9.5 [6.7-13.1]	-.329	10.1 [7.2-12.8]	9.4 [7.0-13.6]	8.7 [5.9-14.9]	7.6 [4.3-14.4]	.484
Platelets (x10^9/L), Median [IQR]							
Admission	203.0 [136.5-299.0]	-.648	241.0 [173.0-320.0]	217.5 [175.5-331.0]	135.0 [72.0-222.0]†,‡	106.0 [61.0-166.0]†,‡	**<.0001**
24 hrs post admission	203.5 [120.0-277.8]	-.643	218.5 [173.3-303.3]	223.0 [163.0-306.8]	136.0 [64.0-233.3]†,‡	91.5 [53.0-150.8]†,‡	**<.0001**
48 hrs post admission	193.0 [102.5-267.0]	-.677	236.0 [170.0-287.0]	214.0 [163.0-301.0]	113.5 [48.8-203.3]†,‡	69.0 [39.5-131.0]†,‡	**<.0001**
72 hrs post admission	193.0 [102.0-285.5]	-.639	222.0 [165.5-303.8]	225.0 [166.3-309.5]	99.0 [42.0-205.8]†,‡	82.0 [42.0-118.0]†,‡	**<.0001**
Coagulation parameters							
PT (seconds), Median [IQR]							
Admission	15.0 [13.2-18.1]	.435	14.8 [13.6-16.8]	14.6 [12.9-16.6]	15.4 [13.3-19.2]	17.7 [13.3-21.3]†,‡	**.014**
24 hrs post admission	14.6 [13.0-17.2]	.399	14.5 [13.2-16.8]	14.4 [12.9-16.5]	14.7 [13.3-18.5]	16.4 [12.8-20.4]	.332
48 hrs post admission	13.9 [12.5-16.0]	.292	14.0 [12.8-15.7]	13.8 [12.3-15.7]	13.9 [12.1-18.1]	14.7 [12.1-17.1]	.706
72 hrs post admission	13.7 [12.3-15.2]	-.126	13.9 [12.8-16.2]	13.6 [12.2-14.9]	13.9 [11.7-16.9]	13.7 [12.5-15.4]	.446
aPTT (seconds), Median [IQR]							
Admission	32.3 [28.6-38.4]	.621	30.3 [27.3-35.5]	31.4 [28.0-37.0]	35.8 [30.2-44.2]†,‡	40.2 [32.8-51.4]†,‡	**<.0001**
24 hrs post admission	33.3 [29.0-39.6]	.553	31.9 [28.2-35.9]	32.8 [28.9-38.6]	35.7 [30.6-43.0]†	39.4 [31.8-45.5]†,‡	**<.0001**
48 hrs post admission	32.1 [28.6-38.3]	.581	30.6 [28.4-35.5]	31.5 [28.4-37.3]	34.5 [30.5-42.6]†,‡	37.5 [30.4-43.9]†,‡	**<.0001**
72 hrs post admission	31.5 [28.4-36.5]	.550	30.5 [28.3-34.0]	31.0 [28.3-35.8]	34.4 [29.8-41.6]†,‡	34.7 [29.0-39.5]†,‡	**.004**
Fibrinogen (g/L), Median [IQR]							
Admission	3.8 [2.5-5.0]	-.565	4.5 [3.0-5.4]	4.0 [3.0-5.1]	3.4 [2.1-4.9]†	2.3 [1.5-3.5]†,‡,§	**<.0001**
24 hrs post admission	4.1 [2.9-5.2]	-.568	4.5 [3.2-5.5]	4.3 [3.3-5.4]	3.7 [2.6-5.2]†,‡	2.7 [1.8-3.8]†,‡,§	**<.0001**
48 hrs post admission	4.4 [3.4-5.4]	-.560	4.6 [4.0-5.8]	4.6 [3.5-5.7]	4.1 [2.1-5.1]†,‡	3.1 [2.3-4.4]†,‡	**<.0001**
72 hrs post admission	4.6 [3.5-5.6]	-.531	4.8 [3.9-6.0]	4.8 [3.8-5.9]	3.6 [2.2-5.2]†,‡	3.7 [2.5-5.0]†,‡	**<.0001**
D-dimer (ng/ml), Median [IQR]							
Admission	4073.5 [2054.8-7759.4]	.377	3788.0 [1925.3-6335.0]	3756.2 [1865.0-6284.8]	5549.3 [1877.6-12276.0]	6261.0 [2464.4-15527.6]†,‡	.143
24 hrs post admission	4485.0 [2147.0-8737.0]	.214	4687.5 [2941.0-10879.3]	4975.8 [1227.0-7251.2]	5064.0 [2015.0-7022.0]	5791.0 [3809.0-15167.0]	.494
48 hrs post admission	5044.1 [2175.9-7394.8]	.333	4145.0 [2783.0-10341.0]	5150.1 [1803.3-7252.7]	4504.5 [2252.3-9001.2]	4729.0 [2784.0-14336.9]	.888
72 hrs post admission	4931.0 [2386.5-7762.7]	.319	4407.0 [2211.0, 9089.0]	4743.0 [2952.0-6919.0]	5275.9 [2733.2-14048.1]	5437.0 [1563.5-19292.0]	.983
Total DIC (n) [%]	58 [17.0%]	-	8 [10.7%]	7 [6.7%]	16 [32.7%]†,‡	27 [57.4%]†,‡,§	**<.0001**
Time to develop DIC (n) [%]							
Admission	28 [8.2%]	-	5 [6.7%]	5 [2.9%]	4 [8.2%]	14 [30.0%]†,‡,§	**<.0001**
24 hrs post admission	13 [3.8%]	-	0 [0.0%]	1 [0.6%]	5 [10.2%]†,‡	7 [14.9%]†,‡	**<.0001**
48 hrs post admission	10 [2.9%]	-	3 [4.0%]	1 [0.6%]	2 [4.1%]	4 [8.5%]‡	**.001**
72 hrs post admission	7 [2.1%]	-	0 [0.0%]	0 [0.0%]	5 [10.2%]†	2 [4.3%]	**<.0001**
Developed DIC ≥24 hrs post admission (n) [%]	30 [8.8%]	-	3 [4.2%]	2 [1.2%]	12 [26.6%]†,‡	13 [39.4%]†,‡	**<.0001**

Table 2. Peripheral blood measurements of absent, mild, moderate and strong NETs formation in ICU patients
* P value for comparisons of vs mild vs moderate vs strong NETs patients collectively. Performed using Kruskall-Wallis test for continuous variables and Chi-squared test for categorical variables. † Significant vs absent NETs patients. ‡ Significant vs mild NETs patients. § Significant vs moderate NETs patients. R correlation with percentage NETs performed using Spearman's rank.

	Crude Odds ratio	*P* value*	Adjusted odds ratio [APACHEII]	*P* value†	AUC	*P* value‡
DIC						
Absent NETs	REF		REF			
Mild NETs	0.274 [0.045-1.677]	.161	0.248 [0.039-1.560]	.137	-	-
Moderate NETs	8.121 [2.143-30.770]	**.002**	7.176 [1.765-29.177]	**.006**	-	-
Strong NETs	14.517 [3.759-56.057]	**<.0001**	13.035 [3.157-53.829]	**<.0001**	-	-
NETs [%]	1.059 [1.041-1.078]	**<.0001**	1.058 [1.039-1.078]	**<.0001**	0.851	**<.0001**
cfDNA	1.001 [1.000-1.001]	.060	1.001 [1.000-1.001]	.118	0.607	.324
MPO	1.001 [0.997-1.004]	.664	1.001 [0.997-1.005]	.689	0.609	.236
DNA-MPO	17.428 [1.976-153.679]	**.010**	9.780 [0.972-98.424]	.053	0.713	.013
IL-1β	0.993 [0.923-1.068]	.845	0.999 [0.920-1.084]	.973	0.588	.272
IL-6	1.000 [1.000-1.000]	.113	1.000 [1.000-1.000]	.139	0.546	.499
TNFα	0.999 [0.994-1.003]	.501	0.999 [0.995-1.003]	.593	0.658	.044
IL-8	1.000 [1.000-1.000]	**.049**	1.000 [1.000-1.001]	.083	0.666	.002
APACHEII	1.144 [1.084-1.208]	**<.0001**	-	-	0.753	**<.0001**
SOFA score	1.435 [1.274-1.616]	**<.0001**	-	-	0.837	**<.0001**
Mortality						
Absent NETs	REF		REF			
Mild NETs	1.385 [0.617-3.109]	.430	1.370 [0.601-3.125]	.454	-	-
Moderate NETs	3.235 [1.284-8.152]	**.013**	2.889 [1.114-7.494]	**.029**	-	-
Strong NETs	3.785 [1.506-9.511]	**.005**	2.995 [1.162-7.720]	**.023**	-	-
NETs [%]	1.020 [1.010-1.030]	**<.0001**	1.016 [1.006-1.026]	**.002**	0.851	**<.0001**
cfDNA	1.000 [1.000-1.000]	.232	1.000 [1.000-1.000]	.532	0.607	.324
MPO	0.998 [.0994-1.002]	.353	0.998 [0.993-1.002]	.261	0.609	.236
DNA-MPO	2.005 [0.430-9.359]	.376	1.432 [0.286-7.161]	.662	0.713	.013
IL-1β	1.008 [.0981-1.035]	.570	1.003 [1.011-1.119]	.804	0.501	.984
IL-6	1.000 [1.000-1.000]	.907	1.000 [1.000-1.000]	.904	0.596	.064
TNFα	0.999 [0.997-1.002]	.671	0.999 [0.996-1.002]	.598	0.511	.846
IL-8	1.000 [1.000-1.000]	.380	1.000 [1.000-1.000]	.563	0.574	.141
APACHEII	1.087 [1.047-1.128]	**<.0001**	-	-	**0.683**	**<.0001**
SOFA score	1.087 [1.017-1.162]	**.014**	-	-	0.604	**.009**

Table 3. NETs formation is an independent predictor of DIC and mortality in critically ill patients

* P value for crude odds ratio to predict DIC and mortality. † P value for adjusted odds ratios to predict DIC and mortality in a multivariable analysis (with patients adjusted for APACHE II scores). ‡ P value for ROC analysis to predict DIC and mortality.

Figure 1

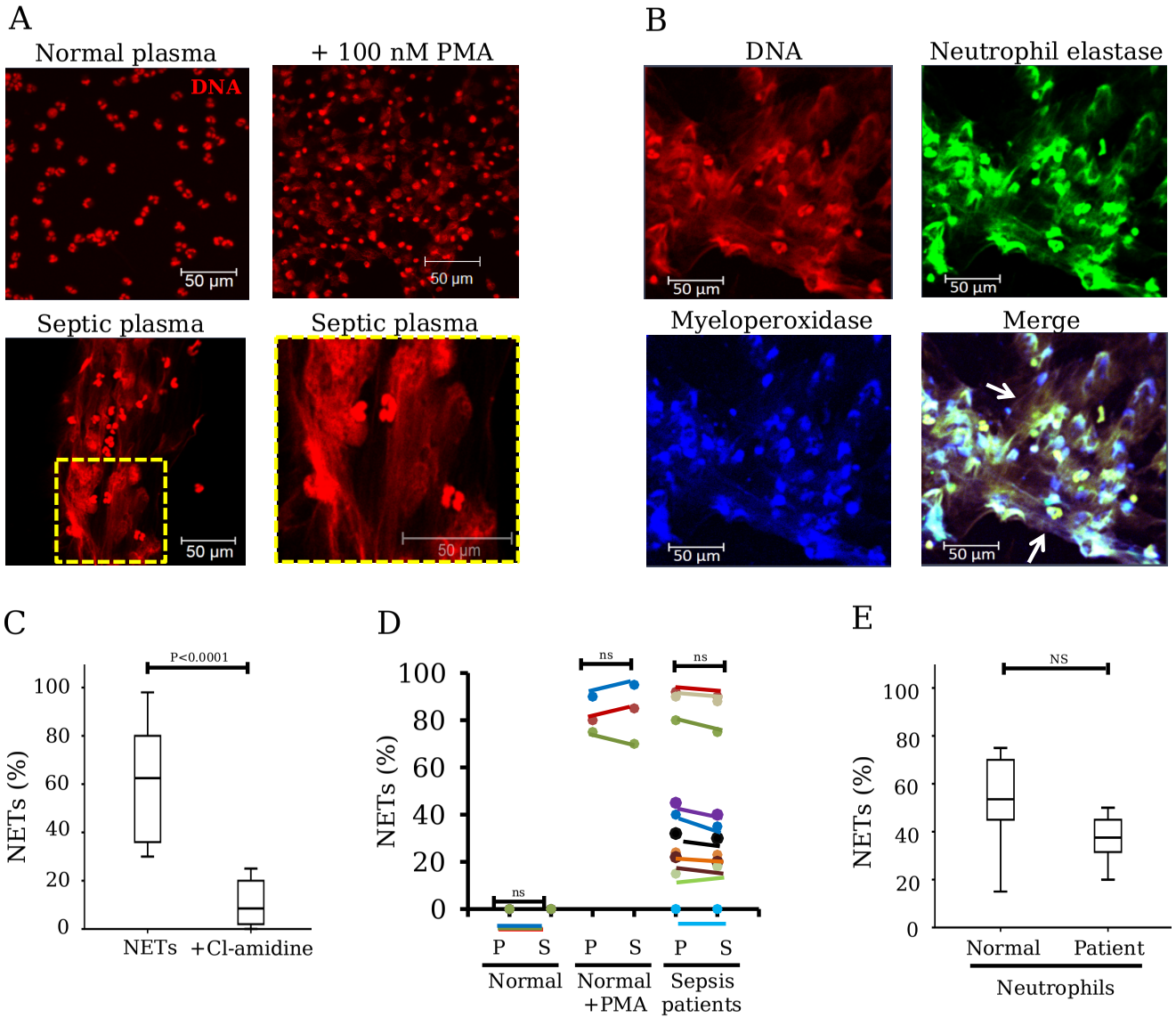

A
Normal plasma
+ 100 nM PMA
DNA
50 µm
Septic plasma
Septic plasma
B
DNA
Neutrophil elastase
Myeloperoxidase
Merge
C
P<0.0001
NETs (%)
NETs
+Cl-amidine
D
ns
NETs (%)
P S P S P S
Normal
Normal +PMA
Sepsis patients
E
NS
NETs (%)
Normal
Patient
Neutrophils

Figure 2

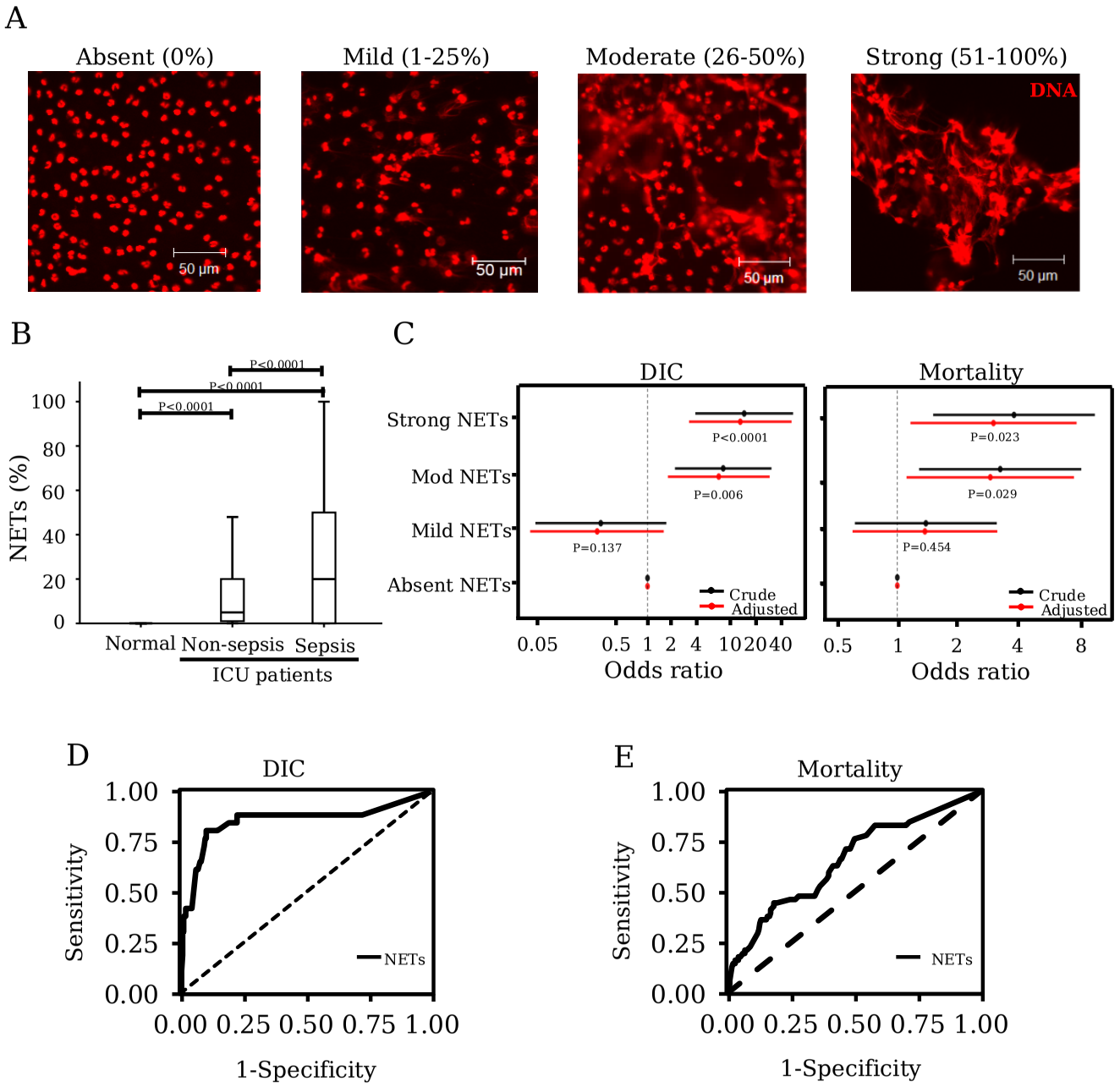
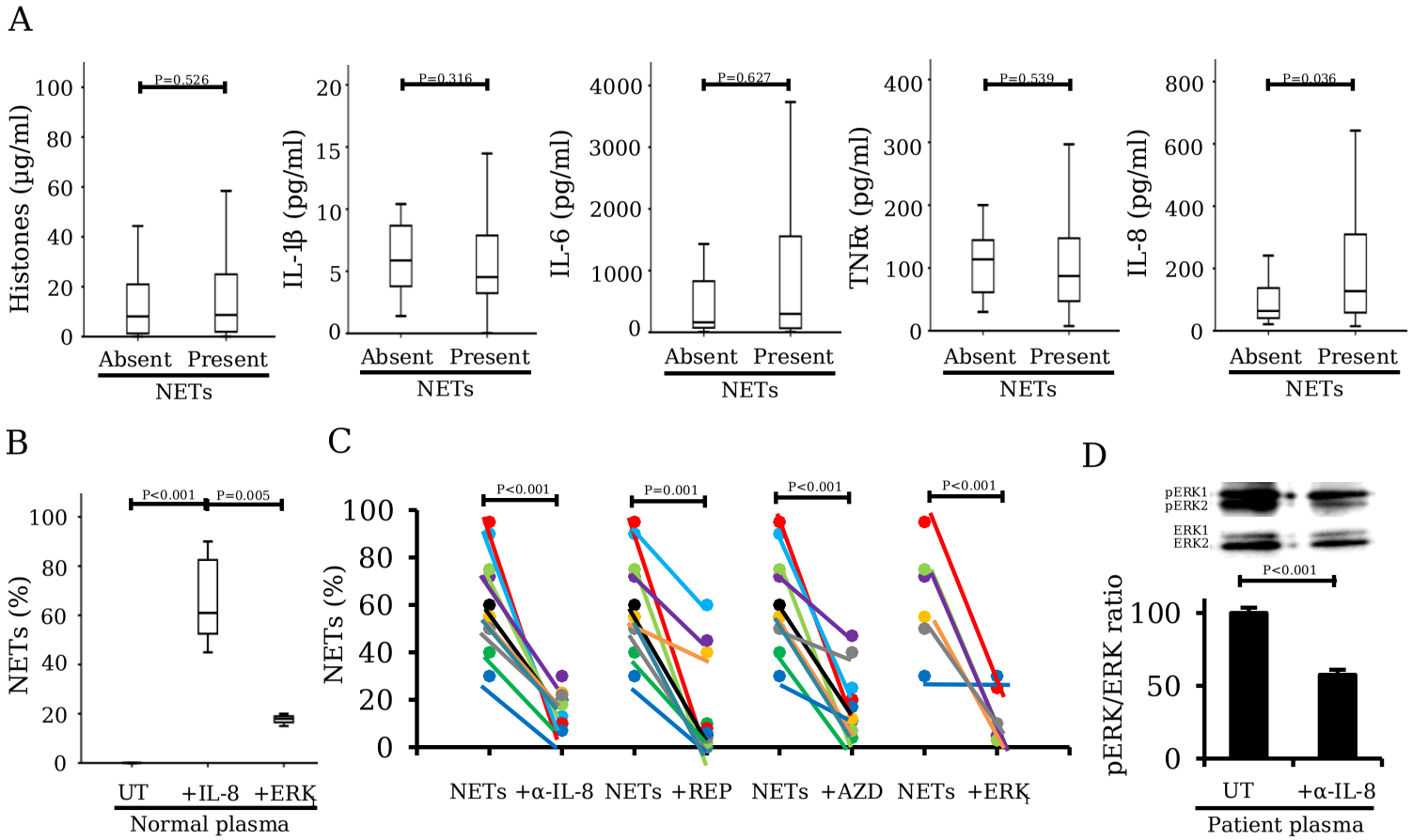

A
Absent (0%)
Mild (1-25%)
Moderate (26-50%)
Strong (51-100%)
DNA
50 µm
B
NETs (%)
P<0.0001
Normal
Non-sepsis
Sepsis
ICU patients
C
DIC
Mortality
Strong NETs
Mod NETs
Mild NETs
Absent NETs
P<0.0001
P=0.006
P=0.137
P=0.023
P=0.029
P=0.454
Crude
Adjusted
Odds ratio
D
DIC
Sensitivity
1-Specificity
NETs
E
Mortality

Figure 3


A
Histones (µg/ml)
P=0.526
Absent Present
NETs
IL-1β (pg/ml)
P=0.316
IL-6 (pg/ml)
P=0.627
TNFα (pg/ml)
P=0.539
IL-8 (pg/ml)
P=0.036
B
NETs (%)
P<0.001
P=0.005
UT +IL-8 +ERK
Normal plasma
C
NETs (%)
P<0.001
P=0.001
P<0.001
P<0.001
NETs +α-IL-8 NETs +REP NETs +AZD NETs +ERK
D
pERK1
pERK2
ERK1
ERK2
P<0.001
pERK/ERK ratio
UT +α-IL-8
Patient plasma

Online Data Supplement

A Novel Assay for Neutrophil Extracellular Traps (NETs) Formation Independently Predicts Disseminated Intravascular Coagulation and Mortality in Critically Ill Patients

Short running head: Monitoring NETosis in critical illness

Simon T Abrams, PhD[1], Ben Morton MBChB, MD[2,3], Yasir Alhamdi, MBChB, PhD[1], Mohmad Alsabani [1], Steven Lane, PhD[4], Ingeborg D Welters, PhD[5,6], Guozheng Wang, MD, PhD[1] and Cheng-Hock Toh, MD[1,7]

eMethods

Patient blood sample collection and measurement

Upon ICU admission, surplus blood samples were collected daily from all patients for the first 96 hours (4 samples per patient: admission, 24 hours, 48 hours and 72 hours post-admission). Plasma was prepared by drawing peripheral blood into citrated vacutainers (4.5 ml 0.109 M + buffered sodium citrate 3.2%, Becton Dickinson, Plymouth, UK) and centrifuged for 20 minutes at 2600xg and 20°C. The resulting plasma supernatant was separated and aliquots stored at -80°C. In some patients, matched sera were also isolated and stored at -80°C.Whole blood platelet, white blood cell and neutrophil counts were measured using a Beckman Coulter DxH800, thrombocytopaenia was microscopically verified. Prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen levels and D-dimers were measured using standard protocols in the coagulation laboratory of the Royal Liverpool University Hospital using an ACL TOP® 700 analyser (Werfen Ltd, UK). A panel of 27 cytokines, chemokines and angiogenic factors (General activation markers: Interleukin (IL)-1β,IL-1ra, IL-2, TNFα, IL-6, IL-15; Chemokines: IL-8, IP-10, MCP-1, MIP-1a, MIP-1b, RANTES; T cell-related: IL-4, IL-5, IL-9, IL-10, IL-12 (p70), IL-13, IL-

17, Eotaxin, INFγ; Bone marrow-derived: IL-7, GM-CSF, G-CSF; Angiogenic factors and endothelial mitogens: bFGF, PDGF-bb, VEGF) were measured by MultiPlex (BioRad) in the plasma of normal donors and critically ill patients upon ICU admission, using a Bio-Plex 100 according to manufacturers' instructions.

Neutrophil isolation

Citrated blood was drawn from healthy donors following written informed consent according to protocol approved by Liverpool University Interventional Ethical Committee (Ref: RETH000685). Neutrophils were purified using two-step gradient centrifugation. Leukocytes were isolated using Histopaque®-1077 (Sigma-Aldrich, UK) and further purified using a Percoll (Sigma-Aldrich) gradient to isolate neutrophils (>90% purity).

NETs specific neutralization

To examine the role of interleukin 8 (IL-8) in NETs formation, normal plasma was supplemented with IL-8 (100 pg/ml) and incubated with normal neutrophils for 4 hours prior to fixation and staining. Neutrophils were also pre-incubated for 10 minutes with IL-8 inhibitors: anti-IL-8 mAb (R&D Systems) (1 µg/ml), Cl-amidine (Cambridge biolabs) (10 µM), Reparixin (Dempé) (250 µg/ml) or AZD5069 (AstraZeneca) (10 nM), or a MAPK signalling inhibitor: U0126 (Sigma) (50 µM) prior to adding plasma.

Comparator NETs assays

Circulating histones levels were determined by Western blot, according to our previous publications.(1-3) Cell free DNA (cfDNA) was fluorescently determined using SYTOX green, as previously described.(4) Briefly, 25µl patient plasma was diluted in a final volume of 100 µl and incubated with SYTOX

green (2 μM final concentration). cfDNA was then determined using a fluorescent plater reader (Ex:488nm/Em:523nm) using known concentrations of genomic DNA as standards. Circulating Myeloperoxidase (MPO) (ThermoFisher) were measured by ELISA according to the manufacturer's instructions. Circulating MPO-DNA complex levels were determined using by ELISA using an anti-MPO (SantaCruz Biotech) capture antibody and anti-dsDNA antibody (ROCHE) as a detector. Citrullinated Histone 3 (Cit-H3) was determined in patient plasma by Western blot using a primary antibody against Cit-H3 (Abcam), data were not included due to non-specificity of the antibody.

Western blot analysis of ERK activation

Western blot analysis was performed on normal healthy neutrophils treated with plasma. To investigate the effect of IL-8 treatment on the activation of ERK neutrophils were treated for 0, 15, 30, 45 and 60 minutes with normal plasma supplemented with IL-8 (100 pg/ml). To establish the role of circulating IL-8 patients in activating ERK, normal heatly neutrophils were incubated without or with pre-treatment with anti-IL-8 mAb (1 μg/ml), prior to treatment with septic patient plasm for 15 minutes. Following treatment, samples were lysed and separated by SDS-PAGE followed by transfer onto PVDF membrane. Following blocking, membranes were probed with 1:1000 anti-pERK antibody (Santa Cruz) overnight and 1:10,000 anti-mouse secondary antibody for 45 mins. Bands were visualised using ECL (Enhanced Chemiluminescence). To ensure equal loading, membranes were stripped using stripping buffer for 30 mins at 50°C and blocked. Membranes were probed with 1:1000 anti-ERK antibody (Santa Cruz) overnight and 1:10,000 anti-rabbit secondary antibody for 45 mins. Bands were visualised using ECL and densitometry performed to determine pERK/ERK ratio.

Multivariate logistical regression analysis

Prior to construction of the multivariate model, we selected variables that could plausibly be associated with DIC and mortality. These variables were tested in univariate analysis to determine their association as are displayed in Table E1. For the multivariate, analysis we selected variables independent from one another with a univariate analysis p value of less than 0.1. Following on from this, we constructed the final multivariate model using a standard stepwise approach, sequentially removing variables with a p value of more than 0.1.

eReferences

1. Alhamdi Y, Abrams ST, Cheng Z, Jing S, Su D, Liu Z, Lane S, Welters I, Wang G, Toh CH. Circulating histones are major mediators of cardiac injury in patients with sepsis. *Crit Care Med* 2015;43:2094-2103.

2. Abrams ST, Zhang N, Manson J, Liu T, Dart C, Baluwa F, Wang SS, Brohi K, Kipar A, Yu W, Wang G, Toh CH. Circulating histones are mediators of trauma-associated lung injury. *Am J Respir Crit Care Med* 2013;187:160-169.

3. Alhamdi Y, Abrams ST, Lane S, Wang G, Toh CH. Histone-associated thrombocytopenia in patients who are critically ill. *JAMA* 2016;315:817-819.

4. Fuchs TA, Kremer Hovinga JA, Schatzberg D, Wagner DD, Lämmle B. Circulating DNA and myeloperoxidase indicate disease activity in patients with thrombotic microangiopathies. *Blood* 2012;120:1157-1164.

	Crude Odds ratio	*P* value*
DIC		
Initial diagnosis		
Sepsis	REF	
Cardiovascular	0.000 [0.000-0.000]	.998
CNS	0.000 [0.000-0.000]	.998
Gastro	0.258 [0.058-1.151]	.076
Renal	0.000 [0.000-0.000]	.999
Respiratory	0.087 [0.019-0.661]	**.018**
Trauma	0.222 [0.064-0.770]	**.018**
Hypotension	2.220 [1.001-4.923]	.050
ARDS (P/F)	1.000 [0.996-1.003]	.945
APACHEII	1.144 [1.084-1.208]	**<.0001**
Bacteraemia	7.625 [3.027-19.205]	**<.0001**
Source of infection		
No infection	REF	
Respiratory	7.843 [2.625-23.431]	**<.0001**
Abdomen	5.337 [1.702-16.736]	**.004**
Neuro	11.091 [2.724-45.150]	**.001**
Other	10.893 [2.953-40.183]	**<.0001**
IL-8	1.000 [1.000-1.000]	**.049**
Age	0.988 [0.967-1.009]	.255
Gender	0.538 [0.249-1.165]	.116
Mortality		
Initial diagnosis		
Sepsis	REF	
Cardiovascular	1.036 [0.442-2.427]	.935
CNS	0.184 [0.024-1.442]	.107
Gastro	0.461 [0.167-1.273]	.135
Renal	0.000 [0.000-0.000]	.999
Respiratory	0.395 [0.155-1.003]	.051
Trauma	0.417 [0.182-0.957]	**.039**
Hypotension	1.113 [0.641-1.933]	.704
ARDS (P/F)	0.998 [0.996-1.001]	**.204**
APACHEII	1.087 [1.047-1.128]	**<.0001**
Bacteraemia	2.109 [1.228-3.623]	**.007**

Source of infection		
No infection	REF	
Respiratory	2.914 [1.421-5.973]	**.004**
Abdomen	1.457 [0.651-3.233]	.355
Neuro	1.166 [0.317-4.280]	.817
Other	2.914 [1.194-7.109]	**.019**
IL-8	1.000 [1.000-1.000]	.380
Age	1.014 [0.997-1.031]	.102
Gender	0.850 [0.498-1.451]	.551

Table E1. Univariate analysis for the prediction of DIC and mortality.
To construct the multivariate model an independent variable was included if univariate analysis indicated a $p<0.1$ and gender (convention). NETosis, IL-8, APACHEII, source of infection (categorical) and gender within the initial multivariate analysis and removed non-significant variables ($p>0.1$) in a stepwise method until all remaining variables were significant. We performed multivariate analysis with the dependent variables of DIC and Mortality. Stepwise regression for DIC, IL-8 ($p=0.117$), source of infection ($p = 0.825$, 0.361, 0.679 and 0.936 respectively) and gender ($p=0.175$) were removed. Stepwise regression for mortality, IL-8 ($p=0.984$), source of infection ($p = 0.814$, 0.348, 0.535 and 0.510 respectively) and gender ($p=0.826$) were removed. Our final models for predicting DIC and mortality are adjusted for APACHE II (Table 3).
* P value for crude odds ratio to predict DIC and mortality.

	Normal	Correlation (R value)	Absent NETs	Mild NETs	Moderate NETs	Strong NETs	P value*
Total number (n)			75	170	49	47	
NETs-related markers							
cfDNA (ng/ml), Median [IQR]	245.70 [154.63-443.21]	-.134	617.9 [378.8-971.3] †	521.8 [237.6-1015.8]	530.3 [367.9-990.5]	496.0 [316.8-1237.4]	.864
MPO (ng/ml), Median [IQR]	12.40 [4.55-35.39]	.327	97.4 [36.8-180.8] †	65.5 [39.4-96.1] †	154.4 [51.7-312.2] †, §	101.1 [33.1-192.2] ‡	.204
DNA-MPO (AU), Median [IQR]	0.97 [0.89-1.11]	.158	0.89 [0.83-1.16]	0.96 [0.84-1.19]	0.94 [0.84-1.08]	0.92 [0.82-1.10]	.982

Table E2. Circulating NETs-related markers in absent, mild, moderate and strong NETs formation in ICU patients

* P value for comparisons of absent vs mild vs moderate vs strong NETs patients collectively. Performed using Kruskall-Wallis test for continuous variables and Chi-squared test for categorical variables. † Significant vs normal controls. ‡ Significant vs absent NETs patients. § Significant vs mild NETs patients. R correlation with percentage NETs performed using Spearman's rank.

	Normal	Correlation (R value)	Absent NETs	Mild NETs	Moderate NETs	Strong NETs	P value*
Total number (n)			75	170	49	47	
General activation							
IL-1β (pg/ml), Median [IQR]	5.65 [4.65-6.34]	-.293	5.86 [3.61-8.98]	5.51 [3.33-7.88]	4.95 [3.72-9.37]	4.51 [2.88-8.62]	.773
IL-1ra (pg/ml), Median [IQR]	177.58 [153.24-247.02]	-.286	1098.86 [318.56-6046.29] †	1025.10 [270.46-5705.21] †	443.08 [190.69-2598.12] †	522.78 [170.83-5580.57] †	.485
IL-2 (pg/ml), Median [IQR]	0.00 [0.00-3.38]	-.297	0.00 [0.00-7.78]	0.00 [0.00-3.53]	0.00 [0.00-0.00] §	0.00 [0.00-4.23]	.176
TNFα (pg/ml), Median [IQR]	96.50 [61.56-125.53]	-.265	113.80 [60.41-158.11]	70.51 [51.83-138.10]	94.47 [43.99-150.35]	100.57 [40.56-171.85]	.663
IL-6 (pg/ml), Median [IQR]	15.15 [12.36-19.52]	.265	161.99 [69.65-1123.54]	445.30 [61.09-1216.67] †	146.80 [40.43-608.98] †	343.86 [85.97-2449.15] †, ‖	.177
IL-15 (pg/ml), Median [IQR]	0.00 [0.00-12.59]	-.241	12.53 [0.00-79.01]	20.91 [0.00-45.54] †	0.00 [0.00-32.41]	24.55 [0.00-55.59] †	.279
Chemokines							
IL-8 (pg/ml), Median [IQR]	29.31 [26.74-55.93]	.529	63.27 [39.28-143.31] †	128.89 [48.47-255.64] †	114.36 [52.76-314.29] †	127.53 [73.50-331.65] †, ‡	.069
IP-10 (pg/ml), Median [IQR]	540.68 [379.22-656.62]	.221	1118.65 [668.75-6877.97] †	1037.15 [527.40-1914.35] †	1632.67 [494.98-7669.59] †	1077.73 [683.36-2954.90] †	.529
MCP-1 (pg/ml), Median [IQR]	4.90 [0.00-19.81]	.235	58.00 [22.67-167.01] †	87.17 [27.21-200.48] †	31.54 [2.35-181.35] §	112.28 [33.96-237.29] †	.172
MIP-1a (pg/ml), Median [IQR]	5.93 [4.14-7.62]	-.230	5.14 [2.98-7.81]	4.37 [2.98-6.09]	4.92 [3.33-7.61]	4.45 [2.36-6.47]	.768
MIP-1b (pg/ml), Median [IQR]	23.64 [14.72-34.15]	-.307	92.75 [71.93-240.96] †	100.55 [61.86-172.69] †	96.33 [68.23-164.86] †	91.12 [60.66-139.34] †	.649
RANTES (pg/ml), Median [IQR]	3142.25 [1278.65-3558.20]	-.560	4690.15 [2372.52-6221.61]	5085.97 [3566.83-7098.21] †	3164.76 [1716.22-4301.35] §	2905.09 [14445.22-4311.39] ‡, §	**<.0001**
T cell-related							
IL-4 (pg/ml), Median [IQR]	6.19 [5.74-7.23]	-.463	6.36 [4.65-8.42]	6.90 [5.30-8.08]	5.86 [4.12-7.94]	5.00 [3.19-7.60] §	.126
IL-5 (pg/ml), Median [IQR]	20.18 [8.99-21.52]	-.608	11.36 [4.79-16.28] †	10.55 [4.79-15.18] †	9.08 [1.90-15.29] †	1.63 [0.00-6.40] †, ‡, §, ‖	**.001**
IL-9 (pg/ml), Median [IQR]	17.54 [14.05-44.76]	-.476	56.71 [23.00-94.63] †	47.95 [26.42-84.40] †	30.53 [21.40-49.81] §	28.25 [18.08-59.16] ‡, §	**.043**
IL-10 (pg/ml), Median [IQR]	17.81 [12.79-26.34]	-.266	31.99 [17.63-136.96]	33.80 [20.01-43.01] †	21.20 [13.85-74.45]	37.54 [16.94-79.03]	.432
IL-12 (p70) (pg/ml), Median [IQR]	8.88 [2.73-26.86]	-.576	16.05 [7.46-21.45]	13.95 [9.04-18.96]	11.09 [6.10-19.36]	7.74 [3.10-13.60] ‡, §	**.004**
IL-13 (pg/ml), Median [IQR]	8.62 [3.77-13.82]	-.528	5.31 [2.14-7.81]	5.15 [3.91-9.85]	5.31 [2.19-8.98]	2.71 [0.11-4.39] †, §, ‖	**.009**
IL-17 (pg/ml), Median [IQR]	29.13 [7.53-33.46]	-.512	39.07 [26.70-71.62]	44.29 [21.38-71.00] †	25.41 [15.91-60.50]	21.74 [5.07-39.31] ‡, §	**.007**
Eotaxin (pg/ml), Median [IQR]	87.97 [62.29-113.57]	-.200	95.45 [58.38-127.69]	93.91 [65.52-123.15]	81.49 [58.91-133.11]	91.30 [61.74-119.68]	.960
INFγ (pg/ml), Median [IQR]	162.57 [124.31-201.07]	-.369	162.97 [119.33-269.09]	146.16 [122.53-205.00]	163.95 [119.55-241.74]	134.46 [79.72-223.60]	.415
Bone marrow-derived							

IL-7 (pg/ml), Median [IQR]	22.05 [15.07-29.12]	-.223	17.72 [8.73-22.44]	14.91 [8.57-23.56]	16.36 [7.48-23.51]	14.38 [5.43-30.81]	.973
GM-CSF (pg/ml), Median [IQR]	0.00 [0.00-0.00]	-.365	90.08 [0.00-177.10] †	66.70 [31.51-143.91] †	0.00 [0.00-55.59] ‡, §	51.78 [0.00-143.58] †, ‖	.006
G-CSF (pg/ml), Median [IQR]	112.28 [108.85-139.09]	-.077	152.70 [112.77-318.01]	226.19 [90.74-821.29]	117.67 [79.94-364.42]	178.59 [76.70-738.95]	**.509**
Angiogenic factors and endothelial mitogens							
bFGF (pg/ml), Median [IQR]	32.85 [10.92-153.67]	-.529	64.80 [50.95-92.73]	77.01 [51.64-99.15]	41.55 [18.20-62.48] ‡, §	48.86 [22.89-76.03] ‡, §	<.0001
PDGF-bb (pg/ml), Median [IQR]	636.75 [152.18-863.18]	-.458	528.68 [147.68-1308.47]	662.11 [284.66-888.55]	455.37 [138.82-825.18]	324.95 [122.72-653.54] §	.196
VEGF (pg/ml), Median [IQR]	11.99 [2.21-62.04]	-.464	78.93 [29.29-112.58] †	65.28 [35.64-107.89] †	54.48 [20.10-98.32] †	49.91 [14.05-82.45] §	.105

Table E3. Circulating cytokine levels in absent, mild, moderate and strong NETs formation in patients on ICU admission.

* P value for comparisons of absent vs mild vs moderate vs strong NETs-formation in ICU patients. Performed using Kruskall-Wallis test for continuous variables and Chi-squared test for categorical variables. † Significant vs Normal controls. ‡ Significant vs absent NETs patients. § Significant vs mild NETs patients. ‖ Significant vs moderate NETs patients. R correlation with percentage NETs in patient samples performed using Spearman's rank.

Figure E1. CONSORT diagram illustrating patients' initial recruitment, excluded groups and final study number

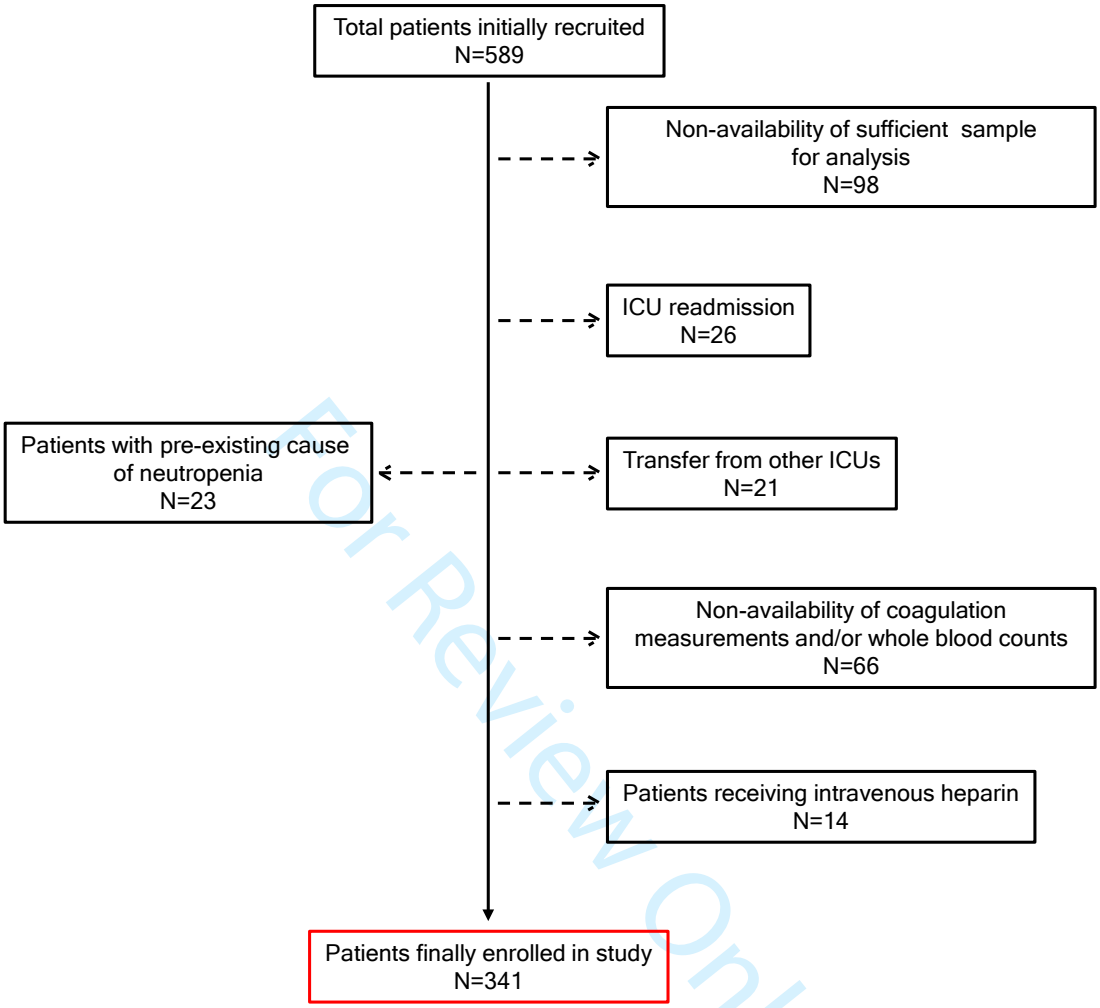


Figure E2. IL-8 induces MAPK activation in neutrophils.
Isolated neutrophils were treated with normal plasma supplemented with IL-8 (100 pg/ml) for indicated time duration. Western blot analysis of ERK activation (pERK/ERK ratio) was then determined relative to T=0.

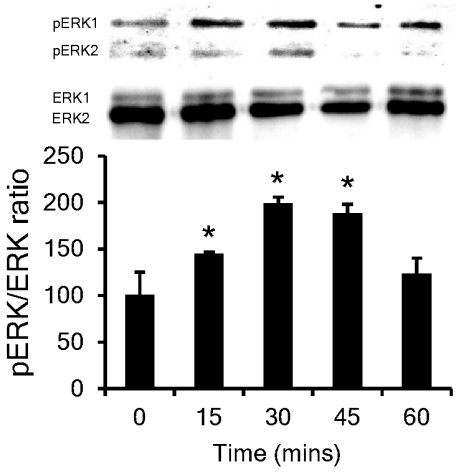


Figure E3. The assay measuring NETs-formation in patient plasma is distinct from NETs-related assays